

16p11.2 600 kb Duplications confer risk for typical and atypical Rolandic epilepsy

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Rolandic epilepsy (RE) is the most common idiopathic focal childhood epilepsy. Its molecular basis is largely unknown and a complex genetic etiology is assumed in the majority of affected individuals. The present study tested whether six large recurrent copy number variants at 1q21, 15q11.2, 15q13.3, 16p11.2, 16p13.11

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and 22q11.2 previously associated with neurodevelopmental disorders also increase risk of RE. Our association analyses revealed a significant excess of the 600 kb genomic duplication at the 16p11.2 locus (chr16: 29.5–30.1 Mb) in 393 unrelated patients with typical ($n = 339$) and atypical (ARE; $n = 54$) RE compared with the prevalence in 65 046 European population controls (5/393 cases versus 32/65 046 controls; Fisher's exact test $P = 2.83 \times 10^{-6}$, odds ratio = 26.2, 95% confidence interval: 7.9–68.2). In contrast, the 16p11.2 duplication was not detected in 1738 European epilepsy patients with either temporal lobe epilepsy ($n = 330$) and genetic generalized epilepsies ($n = 1408$), suggesting a selective enrichment of the 16p11.2 duplication in idiopathic focal childhood epilepsies (Fisher's exact test $P = 2.1 \times 10^{-4}$). In a subsequent screen among children carrying the 16p11.2 600 kb rearrangement we identified three patients with RE-spectrum epilepsies in 117 duplication carriers (2.6%) but none in 202 carriers of the reciprocal deletion. Our results suggest that the 16p11.2 duplication represents a significant genetic risk factor for typical and atypical RE.

INTRODUCTION

Rolandic epilepsy (RE), also known as benign epilepsy with centrotemporal spikes (BECTS), is the most common childhood epilepsy with a prevalence of 0.2–0.73/1000 (i.e. $\sim 1/2500$) (1–3). RE is related to rarer, and less benign epilepsy syndromes, including atypical benign partial epilepsy (ABPE), Landau–Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike-and-waves during sleep (CSWSS) (4–7), referred to as RE related syndromes, or atypical Rolandic epilepsy (ARE) by some authors (8). Together RE and ARE are considered to form the spectrum of epilepsy–aphasia disorders with overlapping clinical characteristics. RE is located on the benign end of this spectrum and has an age-of-onset between 2 and 13 years with a mean onset around the age of seven (9,10). The cardinal clinical features of RE are brief, focal hemifacial or oropharyngeal sensorimotor seizures with speech arrest and secondarily generalized tonic–clonic seizures, which mainly occur during sleep. Classical RE usually takes a self-limiting course with seizures remitting spontaneously during adolescence. Although co-morbidities (e.g. learning and reading disabilities, behavioral issues, attention deficit hyperactivity disorder) are frequently reported in cohorts of children affected by RE, the long-term outcome is excellent (11,12). ABPE, CSWSS and LKS denote more severe forms of early-onset focal childhood epilepsies or epileptic encephalopathies with various additional seizure types, neurocognitive regression, autistic features or a regression of language with speech dyspraxia as in LKS (6,13). RE and ARE share the Electroencephalography (EEG) characteristic of centrotemporal spikes (CTS), blunt spikes of high-voltage and typical morphology. In ARE spikes become generalized during slow wave sleep and their morphology, location and background activity may vary to some extent (5,8). The CTS EEG trait is not entirely specific to RE, as it is also found in 2–4% of healthy children (14,15), in 10–28% of children with autism spectrum disorder (ASD) (16–18) and in the Fragile-X-Syndrome (19).

The underlying etiologies of RE remain largely unknown although a genetic basis is postulated. Multiplex family studies suggested an autosomal dominant inheritance of the EEG trait CTS (20,21). However, several other arguments, among them the distribution of seizure-risk in relatives of patients with RE, argue for a complex mode of inheritance (22). Linkage studies identified loci for CTS on 15q14 (LOD 3.56) and 11p13 (LOD 4.30) (23,24). Fine mapping of the latter locus revealed an association with the *ELP4* gene, but causative mutations have not yet

been identified (24). Markers linked to chromosome 16p12–11.2 (LOD 3.68) were found in one family affected by RE with paroxysmal exercise-induced dystonia and writer's cramp (25), while disease-associated variants in the *SRPX2* gene were found in a family with X-linked RE, oral and speech dyspraxia and intellectual disability and in one patient with perisylvian polymicrogyria and Rolandic seizures (26). Rare variants in *KCNQ2* and *KCNQ3* were identified in a small number of patients with RE (27). Recently others and we showed that genomic alterations in *GRIN2A*, *RBFOX1*, *RBFOX3* and *DEPDC5* are genetic risk factors for idiopathic focal epilepsies with CTS, with and without language dysfunction (28–32).

Copy number variants (CNVs) are an important source of structural genomic variation. Whereas many CNVs, especially non-recurrent ones, are not necessarily related to a clinical phenotype, six prominent recurrent CNVs (1q21, 15q11.2, 15q13.3, 16p11.2, 16p13.11 and 22q11.2) are strongly associated with seizures and a range of neurodevelopmental and neuropsychiatric disorders including autism (33–38). One of the characteristic features of these recurrent CNVs is their remarkable phenotypic variability suggesting a shared genetic basis of the above diseases. The 16p11.2 600 kb BP4–BP5 microdeletion (OMIM #611913) is associated with ASD, obesity and intellectual disability with and without epilepsy, whereas the reciprocal duplication (OMIM #614671) is linked to schizophrenia, microcephaly, intellectual disability and being underweight (39–45). Recurrent microdeletions at 15q11.2, 15q13.3 and 16p13.11 have been identified as important risk factors for genetic generalized epilepsy (GGE) accounting for $\sim 1\%$ of these patients but are also associated with a range of other neuropsychiatric symptoms (33,34,46). Similarly, the 16p11.2 duplication was recently reported in two patients with RE with atypical electro-clinical features (47). These pleiotropic effects and the associations between recurrent CNVs and GGE prompted us to investigate systematically the frequency of these six recurrent CNVs and novel large CNVs in a cohort of children with RE or ARE. In this study one we report a significant association of the recurrent 16p11.2 microduplication and a borderline association of the 15q11.2 duplication with typical and atypical RE. The 16p11.2 association was independently reproduced by phenotyping a large cohort of carriers of recurrent 16p11.2 structural variations, ascertained through learning impairment and behavioral disturbances, where the duplication but not the reciprocal deletion was associated with RE/ARE.

Table 1. Recurrent CNVs in 281 RE/ARE probands and 1512 Controls

CNV locus	Position (Mb) ^a	CNV	Cases (<i>n</i> = 281) <i>n</i> detected	PID	Phenotype	Controls (<i>n</i> = 1512) <i>n</i> detected	OR	<i>P</i> -value uncorrected	<i>P</i> -value Bonferroni ^b corrected
15q11.2	22.8–23.3	DEL	1	GGRE14	RE	0	–	ns	0.17
		DUP	6	AVRE10	RE	8	4.1	0.014*	
				AVRE11					
				AVRE12					
				AVRE13					
				AVRE14					
15q13.3	30.9–32.5	DEL	1 ^c	GGRE15			–	ns	
		DUP	0	AVRE15	ARE	1	–	ns	
16p11.2	29.5–30.1	DUP	4	F1-II.1	3x RE	1	21.8	0.0026*	0.03*
				F2-III.1	1x ARE				
				F3-II.1					
				F4-II.1					
16p13.11	14.8–16.4	DEL	0			2	–	ns	
		DUP	0			1	–	ns	
22q11.2	19.1–22.2	DUP	1	AVRE04	RE	0	–	ns	
	18.9–20.4	DUP	1	F3-II.1	RE				

^aApproximate genomic coordinate according to hg19.^bCorrected for 12 comparisons (for six loci with two CNV states each).^cDeletion is greater than the classical recurrent 1.4 Mb deletion.

ARE, atypical Rolandic epilepsy; RE, Rolandic epilepsy; ns, not significant.

CNVs showing significant *P* values <0.05 are marked with *.

RESULTS

CNV detection

In our discovery cohort of 281 unrelated patients we identified CNVs at four of the six investigated recurrent candidate loci (15q11.2, 15q13.3, 16p11.2 and 22q11.2) (Table 1) as well as other CNVs (Table 2).

We found a significant enrichment of 16p11.2 duplications in RE/ARE patients when compared with controls. Four patients (1.42%) carried a ~600 kb 16p11.2 microduplication compared with only one in 1512 controls (0.07%) [Fisher's exact test, *P* = 0.0026, odds ratio (OR) = 21.8; 95% confidence interval (CI): 2.14–1068.36, *P* (after Bonferroni correction) = 0.03] (Table 1). All four tentative 16p11.2 duplications were validated by Taqman quantitative real-time PCR and custom array-CGH and could be established as the typical 600 kb sized 16p11.2 duplication with the classical breakpoint boundaries at BP4 and BP5 hotspots (44). To confirm our findings we subsequently screened the 16p11.2 locus in an independent sample of 112 patients with RE/ARE. This screen revealed one 16p11.2 microduplication spanning 600 kb in size between breakpoints BP4/BP5 (Fig. 1) which was validated by quantitative real-time PCR. Joint analysis provided significant evidence for an association of the 600 kb 16p11.2 duplication with RE/ARE (5/393 cases versus 1/1512 controls, Fisher's exact test, *P* = 0.0018, OR = 19.43, 95% CI: 2.17–916.84). We also identified one additional RE patient in our study sample of 281 RE/ARE patients carrying a smaller partial 16p11.2 duplication (~110 kb in size; hg19 chr16:29650000–29760000). This atypical rearrangement starts at BP4 and encompasses the genes *SPN*, *QPRT* and *C16orf54* (Supplementary Material, Fig. S1; of note this smaller 110 kb duplication was not considered for the statistical analysis).

The present association of the 16p11.2 duplication with RE/ARE is further supported by a recent genome-wide CNV study that

observed 16p11.2 duplications in two out of 47 RE patients [47]. To evaluate the previously reported prevalence of 16p11.2 duplications in the German population we examined array data from an additional cohort of 2256 healthy individuals from Germany (KORA, PopGen) unscreened for epilepsy (52) but did not detect any 16p11.2 rearrangements. This is in accordance with previous data reporting a prevalence of the 16p11.2 duplication of 0.05% in the European population (31 duplications out of 58 635 tested individuals) (45). A meta-association analysis of all available data (including two published patients) (47) strengthens the association even further as seven 600 kb duplications carriers out of 440 RE/ARE patients can be contrasted against 32 carriers among 65 046 European population controls (Fisher's exact test *P* = 7.53×10^{-9} , OR = 32.8, 95% CI: 12.16–76.38). To explore whether the 16p11.2 duplication confers risk to a broad range of common epilepsy syndromes, we screened 1408 European patients affected by GGE and 330 German patients diagnosed for mesial temporal lobe epilepsy for 16p11.2 duplications using high-density SNP arrays, but detected none (Fisher's exact test *P* = 2.1×10^{-4}).

We also identified a nominally significant increase of duplications at the 15q11.2 locus (approximate genomic coordinates according to hg19 chr15:22750000–23350000) in our discovery cohort. Six patients displayed the duplication in comparison to 8 out of 1512 controls (Fisher's exact test, *P* = 0.014, OR = 4.1; 95% CI: 1.16–13.59). This borderline association did not remain significant after Bonferroni correction for multiple comparisons.

Clinical data and segregation analysis

Patients harboring the 16p11.2 duplication either suffered from typical RE (*n* = 5) or atypical RE (*n* = 1) (Table 3). Three

Table 2. Heterozygous CNVs >500 kb in 281 RE/ARE patients

Case ID	Gender	Cytoband	Coordinates ^a (hg19/Build37)	Size (kb)	CNV	Deleted/duplicated genes ^b	Carriers found in controls (n = 1512)	Previously reported ^c
AVRE01	Female	1q31.2-q31.3	Chr1:193694880–195909031	2214	dup	<i>None</i>	0	
GGRE01	Male	1p36.32	Chr1:4529544–5043734	514	del	<i>AJAPI, BC037321</i>	0	
GGRE02	Male	2p24.1	Chr2:21683285–23585786	1903	del	<i>AK090620</i>	0	
GGRE03	Male	2p22.3	Chr2:32659489–33235324	576	dup	<i>BIRC6, MIR558, TTC27, LOC285045, LOC100271832, LTBP1</i>	4	
GGRE04	Male	3q24	Chr3:143178525–145790803	2612	dup	<i>SLC9A9, NHE9, C3orf58, PLOD2</i>	0	
AVRE02	Male	3q29	Chr3:195460549–196096451	636	dup ^d	<i>MUC20, MUC4, TNK2, AK127609, SDHALP1, TFRC, BC151150, BC144580, ZDHHC19, OSTalpha, AF088041, PCYT1A, TCTEX1D2, TM4SF19, AK124973, OCTM4, UBXN7</i>	0	Recurrent Hotspot 3q29duplication
AVRE03	Male	5p15.2	Chr5:12543792–13312470	769	del	<i>TAG1</i>	0	
GGRE05	Female	5p14.3	Chr5:20886878–21437412	551	dup	<i>AK093362, LOC728411</i>	0	
GGRE06	Female	5q15	Chr5:96507017–97053083	546	del	<i>RIOK2</i>	0	(48)
AVRE04	Male	7p11.2	Chr7:57256128–57878853	623	dup	<i>DQ598473, ZNF716, L37717</i>	1	
GGRE07	Female	8q13.3	Chr8:70931383–71738054	807	del	<i>PRDM14, NCOA2, TRAM1, LACTB2, XKR9</i>	0	
AVRE04	Male	8q21.13-q21.2	Chr8:82517136–85021548	2504	del	<i>IMPA1, SLC10A5, ZFAND1, CHMP4C, SNX16, BC038578</i>	0	(49)
AVRE05	Female	10q21.1	Chr10:56288865–56840255	551	dup	<i>PCDH15</i>	0	(50)
GGRE08	Male	11q11-q12.1	Chr11:54695706–55849535	1154	dup	<i>TRIM48, AB231737, OR4A16, OR4A15, OR4C15, OR4C16, OR4C11, OR4P4, OR4S2, OR4C6, OR5D13, OR5D14, OR5L1, OR5D18, OR5L2, OR5D16, SPRYD5, OR5W2, OR5I1, OR10AG1, OR7E5P, OR5F1, OR5AS1</i>	0	
GGRE09	Male	12q24.33	Chr12:129806980–130479794	673	dup	<i>TMEM132D</i>	2	
GGRE10	Male	14q22.2-q22.3	Chr14:54629941–56027815	1398	del	<i>CDKN3, AY257479, UNQ155, CNIH, GMFB, CGRRF1, SAMD4A, AK096898, KIAA1053, GCH1, WDHD1, SOCS4, MAPK11P1L, LGALS3, DLGAP5, FBXO34, KIAA0831, TBPL2, C14orf33</i>	0	
GGRE11	Male	16q12.1	Chr16:48889177–49469134	580	dup	<i>CBLN1, C16orf78</i>	0	
GGRE12	Female	17q12	Chr17:34815551–36182400	1367	dup	<i>ZNHIT3, MYO19, PIGW, GGNBP2, DHRS11, MRM1, BC084573, LHX1, AATF, ACACA, C17orf78, TADA2A, TADA2L, DUSP14, SYNRG, APIGBP1, DDX52, HNF1B</i>	0	Recurrent Hotspot 17q12 duplication (51)
AVRE07	Female	17q25.1	Chr17:71834171–72622965	789	dup	<i>RPL38, MGC16275, TTYH2, Z49982, DNAI2, BX648926, KIF19, BTBD17, GPR142, GPRC5C, AK126429, CD300A, CD300LB, CD300C, CD300LD, C17orf77, CD300E</i>	2	
AVRE08	Male	18p11.21	Chr18:13760290–14988113	1228	dup	<i>RNMT, MC5R, MC2R, ZNF519, BC034578, ANKRD20A5, AX747360, DQ578597, DQ587539, DQ590589, DQ583161, DQ596563, DQ596206, LOC284233, CXADRP3, POTEC, ANKRD30B</i>	0	
GGRE13	Male	22q13.32-q13.33	Chr22:49396413–49988815	592	dup	<i>BC033837</i>	0	
AVRE09	Male	Xp22.31	ChrX:6470011–8135053	1665	dup	<i>HDHD1A, STS, VCX, PNPLA4, MIR651</i>	0	(49)

^aStart and end positions of the CNVs were assessed with PennCNV.^bGenes are based by PennCNV-boundaries and ENSEMBL database. Genes which are partially affected are included.^cCNV overlaps partly with CNV previously reported in epilepsy CNV studies. Reference is given when CNVs are from the same type.

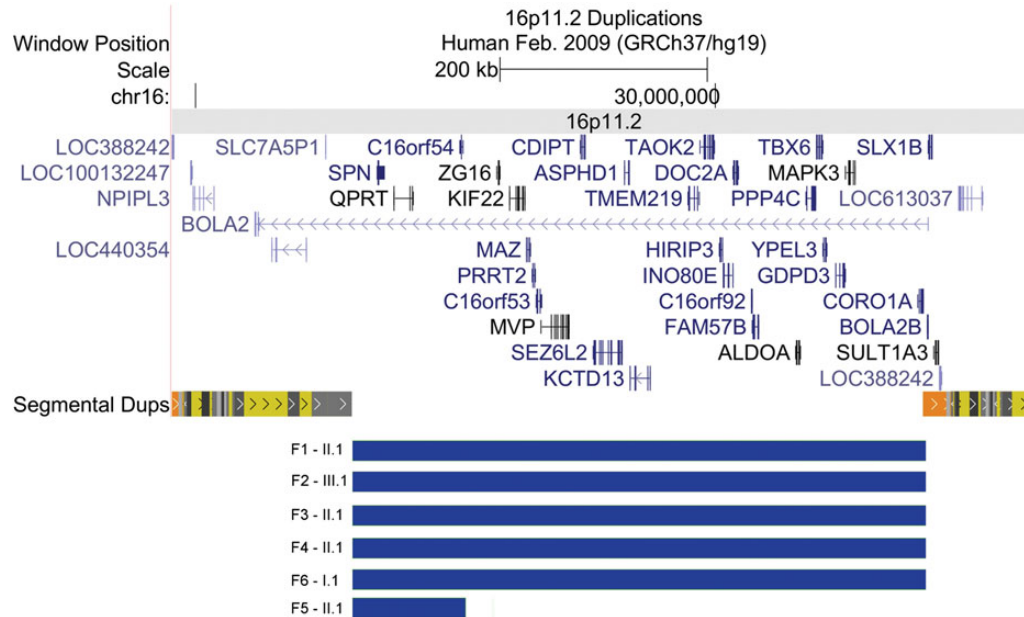


Figure 1. 16p11.2 duplications. GRCh37/hg19 region on chromosome 16p11.2. The extent of the BP4–BP5 16p11.2 duplications found in the discovery cohort is compared with that of the small atypical duplication. The duplication is flanked by low-copy repeat segmental duplications depicted by yellow, orange and gray bars. Note that only the unique sequences of the duplications are shown.

patients (F1-II.1, F3-II.1 and F5-II.1) had in addition a history of febrile seizures, which can be expected in ~20% of children with RE or ARE (53). All patients diagnosed with typical RE showed normal development and their neuropsychological assessments fell within the normal range. Only patient F1-II.1, diagnosed with ARE/LKS, presented with severe developmental delay. For individuals F3-II.1 and F6-I.1 calculations of the body mass index (BMI) were possible, revealing severely to moderately reduced BMIs (F3-II.1: 13.31; F6-I.1: 18.6) consistent with previous reports (45). No data were available on the BMIs of the remaining duplication carriers and on head circumferences of all patients. The duplications were inherited in all four cases where testing was possible (Fig. 2) with a maternal transmission in three of them. The smaller 110 kb atypical duplication was inherited from the father who himself had a history of febrile seizures (Family 5). While the classical 600 kb duplication co-segregated with RE in one family (Family 2), two other families displayed reduced penetrance with reportedly unaffected mothers carrying the duplication (F1-I.2 and F4-I.2). We could not determine whether these mothers did exhibit the RE-characteristic EEG trait during their childhood. Family 1 is interesting as it demonstrates the variability of the phenotypes in the duplication carriers, as the index case (F1-II.1) was affected by severe ARE/LKS, while his younger sister only displayed CTS in EEG (F1-II.3). Notably, the third sibling in this family (F1-II.2) presented the EEG trait without being a duplication carrier (Fig. 2). This family is not only affected by the 16p11.2 duplication but, as recently reported, also carries a novel p.C231Y *GRIN2A* mutation (28). Whereas the *GRIN2A* variation segregates with the CTS trait, the 16p11.2 duplication does not seem to be necessary for the electro-clinical component of the phenotype. In Family 4, we detected in addition to the 16p11.2 duplication a novel missense variation of unknown significance in *DEPDC5*, a gene, which was recently identified in

genetic focal epilepsies (54,55) and also identified by us to be mutated in genetic focal childhood epilepsies (32). In this family the 16p11.2 duplication was inherited from the unaffected mother and the *DEPDC5* variant from the unaffected father, resulting in a potentially increased mutational load in the child. In individual F3-II.1 we identified a second large duplication (~1.5 Mb) on chromosome 22q11.21. Of note, this duplication is shorter than the classical recurrent 22q11.21 duplication, which is 3 Mb in size (OMIM #608363). We did not identify other potentially deleterious variants in recently identified candidate genes (e.g. *RBFOX1* and *RBFOX3*) in the 16p11.2 duplication carriers.

Genotype–phenotype correlations in the 16p11.2 rearrangement cohort

To further specify the association between RE/ARE and 16p11.2 duplications, we examined a large cohort of 319 children (≤ 18 years) with 600 kb BP4–BP5 rearrangements (202 deletions and 117 duplication carriers) from the 16p11.2 European consortium. We detected an equal rate of 18% of patients with epilepsies (combining unspecified, focal, generalized epilepsies and infantile spasms) among the duplication carriers ($n = 22/117$) and deletion carriers ($n = 37/202$) (Supplementary Material, Table S1). In the duplication group, three individuals were diagnosed with RE/ARE (Supplementary Material, Table S2). One patient presented with typical RE (short nocturnal motor facial seizures and centrottemporal spikes activated by sleep). He also suffered from reading and spelling difficulties and was diagnosed with an attention deficit disorder. The second patient displayed the same electro-clinical features but, in addition, also presented with a delay of language, a behavioral disorder, a borderline IQ (75) and an early age of onset (2 years of age). He was therefore diagnosed as atypical RE. The third patient displayed

Table 3. Individuals with 16p11.2 duplication and their electro-clinical features

PID	Index-case	Phenotype	Gender	Age at onset	Seizures	EEG	MRT/ CT	Neuropsychological development	Language development	Family History/ relationship	16p11.2 duplication/size
Discovery cohort											
F1-II.1	Yes	ABPE/ LKS	M	1.0 years/ 3.0 years	FS/GTCS	ESES	Normal	Severe delay	Severe delay, speech regression	Sibling of F1-II.3	~600 kb
F1-II.3		EEG only	F	7 years	No seizures	CTS	Normal	Mild delay	Mild delay	Sibling of F1-II.1	~600 kb
F2-II.2		RE	F	?	Nocturnal TCS	CTS	Normal	Normal	Normal (dyslexia)	Mother of F2-III.1	~600 kb
F2-III.1	Yes	RE	M	2.5 years	Nocturnal TCS, Rolandic seizures	CTS	Normal	Normal	Normal	Positive/ son of F2-II.2	~600 kb
F3-II.1	Yes	RE	M	0.5/5 years	FS/nocturnal TCS	CTS	Normal	Normal	Normal	Negative	~600 kb
F4-II.1	Yes	RE	F	5 years	TCS	CTS/transient ESES	Normal	Normal	Normal	Negative	~600 kb
F5-I.1		FS	M	?	FS	?	?	?	?	Father of F5-II.1	~110 kb
F5-II.1	Yes	RE	F	1.6/5.5 years	FS/TCS, Rolandic seizures	CTS	Normal	Normal	Normal	Positive/daughter of F5-I.1	~110 kb
Confirmation cohort											
F6-I.1	Yes	RE	F	6 years	Nocturnal TCS, staring	CTS	Normal	Normal	Normal	Negative	~600 kb

Clinical characteristics of 16p11.2 duplication carrier. In total five unrelated 600 kb 16p11.2 duplication carrier and one unrelated 110 kb 16p11.2 duplication carrier were identified. The table lists in addition to the six index-cases their duplication carrying relatives.
RE, Rolandic epilepsy; ABPE, atypical benign partial epilepsy of childhood; FS, tonic clonic seizures; ESES, electrical status epilepticus during slow sleep; CTS, centropetential spikes; LKS, Landau-Kleffner syndrome.

an ARE-epileptic encephalopathy (initially CSWSS, but eventually language regression and ASD traits which led to the diagnosis of LKS). An additional (fourth) patient had only CTS activated by sleep without seizures. In the deletion group, a single patient had CTS activated by sleep without seizures but none suffered from RE/ARE. Hence RE/ARE occurred only in duplication carriers (2.6% $n = 3/117$) but not in deletion carriers ($n = 0/202$) (Fisher's exact test, $P = 0.05$). Although it was not possible to assess the exact prevalence of RE/ARE in the complete cohorts screened (i.e. including cases without 16p11.2 rearrangements), we note that RE/ARE features are enriched ~25-fold in the 16p11.2 duplication cohort as compared with the general population given an RE-prevalence of 0.05% (45).

Other structural rearrangements

To investigate whether other large CNVs beyond the above six candidate regions were present in our RE/ARE patients we performed a genome-wide screen for CNVs larger than 500 kb in all 281 individuals of the discovery cohort. In particular, we were interested whether 16p11.2 duplication carriers might display additional large CNVs. We found 23 additional CNVs in the 281 RE/ARE patients screened (Table 2). Six of these CNVs were partially overlapping with CNVs previously reported in patients with epilepsy (48–51,56,57). With the exception of one duplication mapping to chromosome 12q24.33, which was identified in two patients and in two out of 1512 controls from the discovery cohort, all other CNVs occurred as single events in our dataset. Apart from individual F3.II.1, no other 16p11.2 duplication carrier harbored an additional large CNV.

DISCUSSION

We report a highly significant association of the recurrent 16p11.2 microduplication with RE/ARE spectrum epilepsies. We detected the 600 kb 16p11.2 duplication in 1.3% of RE-patients (i.e. in five unrelated individuals: in a sixth unrelated patient we discovered a smaller 110 kb atypical rearrangement, which was not included in the statistical analysis). This corresponds to an enrichment of >25-fold when compared with the prevalence in the general population (0.05%) (45). During the preparation of this manuscript, a genome-wide CNV analysis revealed hemizygous 16p11.2 duplications in two out of 47 RE/ARE patients (47). This CNV report strengthens our association claim. Meta-analysis of the joint RE/ARE cohorts and the assembly of multiple publicly available control cohorts highlights a significant excess of the 600 kb 16p11.2 duplication in RE/ARE patients (7/440 RE/ARE patients versus 32/65 046 controls, $P = 7.53 \times 10^{-9}$). Notably, the observed OR of 32.8 might be overestimated given that our cohorts partly included RE/ARE patients (98 of 393 patients) with a positive family history. However, such a bias seems to be small in this study as the majority of patients (75%) were enrolled irrespective of their family history and as the frequency of the duplication carriers was similar in the multiplex and singly ascertained cohorts (4/295 and 2/98 respectively). Given that we observed a significant enrichment of 16p11.2 duplications in RE/ARE patients but not in other common epilepsy syndromes, such as GGEs or mesial temporal lobe epilepsies (mTLEs), our findings implicate

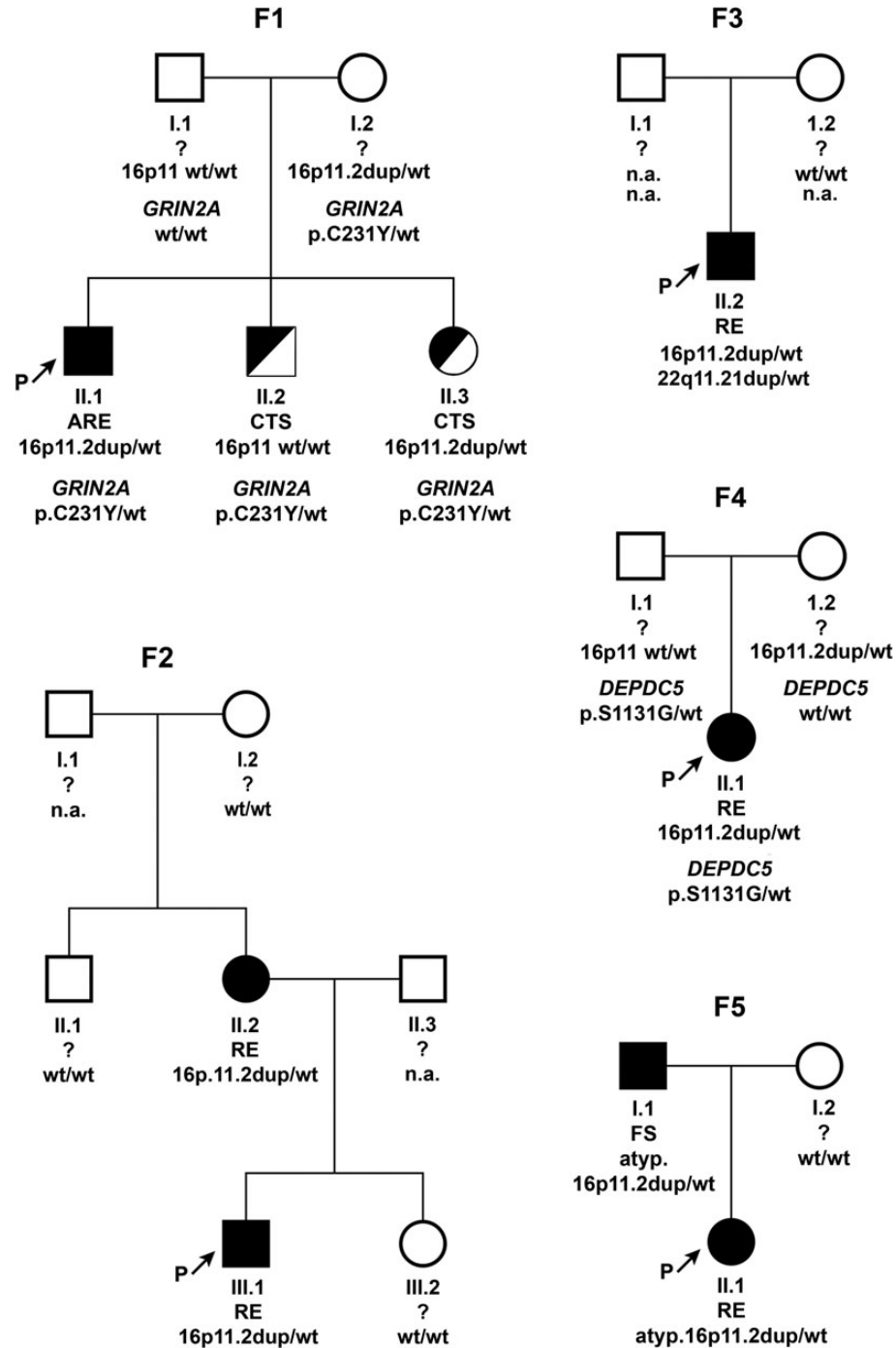


Figure 2. Pedigrees of 16p11.2 microduplication carriers. Familial segregation of 16p11.2 microduplications. Index cases are depicted by the letter 'P' and an arrow. Individuals diagnosed with RE/AE and/or febrile seizures (FS) are represented as filled black symbols, individuals with centrotemporal spikes (CTS) only are depicted by semi-open symbols, unaffected family members are indicated by open symbols. Question marks denote unaffected family members in whom a possible EEG-phenotype in childhood cannot be excluded; RE, Rolandic epilepsy; ARE, atypical Rolandic epilepsy;

a selective contribution of the 16p11.2 duplication to the genetic variance of RE/AE. Future studies on larger cohorts are required to delineate the phenotypic spectrum of epilepsies associated with the 16p11.2 duplication.

Although the effect size appears considerable, the penetrance is incomplete as illustrated by the presence of clinically apparently unaffected duplication carriers in our families (with the caveat

that mild childhood phenotypes might have been missed or not reported in adult carriers). Likewise, the severity and expressivity of the phenotypes varied considerably between affected members of the same family. Similar observations have been made with other CNVs that are nevertheless firmly established as risk factors for epilepsies (33). One remarkable feature of the 16p11.2 rearrangements is the wide diversity of the associated

phenotypes. The BP4–BP5 duplication and its reciprocal deletion were previously associated with schizophrenia, micro-/macrocephaly and underweight/obesity mirror phenotypes as well as impairment in cognitive performance (39,40,42,43,45).

The 16p11.2 duplication is also a known risk factor for ASD (58), emphasizing the often discussed link between autism and epilepsy. Autistic features are frequently detected in severe forms of epilepsy associated with mental retardation and language regression (59–61) and conversely the prevalence rates of epilepsy in ASD is increased up to 46% (62). In our RE/ARE cohort, neither the five 600 kb 16p11.2 duplication carriers nor the 110 kb duplication proband exhibited ASD traits, while a single individual from the 16p11.2 rearrangement cohort, diagnosed with LKS, displayed the autistic signs typical for this syndrome. Our present findings raise questions which warrant further studies as the data described here do not allow a definite distinction between these possibilities. How does the 16p11.2 duplication variation predisposes to both RE and autism? Is it by increasing the risk to a combined phenotype (e.g. by affecting the same gene(s) located in both intertwined disease pathways)? Or are eventually the two phenotypes just coincidentally related to each other due to the involvement of separate genes within the duplication.

In the present report we extend further the list of neurodevelopmental disorders associated with the recurrent 16p11.2 CNV with the full spectrum of the RE/ARE phenotype ranging from mild RE cases to severe forms of ARE with epileptic encephalopathies. Fitting with clinical observations which have long suggested a shared etiology between RE and a wide spectrum of other neuropsychiatric syndromes and symptoms (e.g. cognitive deficits, language disorders, ASD, attention deficit hyperactivity disorder) (12,63) we have identified the 16p11.2 duplication as one shared genetic susceptibility factor. Given the phenotypic variability modulating genetic variants should be expected and consistently such additional genetic factors were identified in three of our families. The exact phenotype of a 16p11.2 duplication carrier would thus depend on the genetic background and additional environmental factors. Elucidating their roles warrants future research.

The 600 kb long chromosomal region between BP4 and BP5 on 16p11.2 contains 28 ‘unique’ genes (*SPN*, *QPRT*, *C16orf54*, *ZG16*, *KIF22*, *MAZ*, *PRRT2*, *PAGR1* (aka *C16orf53*), *MVP*, *CDIPT*, *CDIPT-AS*, *SEZ6L2*, *ASPHD1*, *KCTD13*, *TMEM219*, *TAOK2*, *HIRIP3*, *INO80E*, *DOC2A*, *C16orf92*, *FAM57B*, *ALDOA*, *PPP4C*, *TBX6*, *YPDL3*, *GDPD3*, *MAPK3*, *CORO1A*) and multiple copies of *BOLA2/2B*, *SLX1A/1B*, *SULT1A3/4* and *NPIPL3* (Fig. 1). Most of these genes are expressed in the brain and might thus contribute singly or jointly to the etiology of RE and related disorders. Several studies on model organisms have attempted to narrow down the dosage sensitive genes for the observed brain-related traits. Haploinsufficiency of *ALDOA* and *KIF22* have been related to changes in brain morphology in zebrafish (64), while alterations in head size anticorrelated with levels of *KCDT13*, *MAPK3* and *MVP* in teleosts and rodents (65). One particularly interesting candidate is the *PRRT2* gene with a putative role in synaptic vesicle functioning, as it has been associated with a range of different childhood epilepsies. Mutations in *PRRT2* are, for instance, the main cause of benign familial infantile seizures (BFIS) and other infantile epilepsy syndromes as well as paroxysmal kinesigenic dyskinesia

(66–70). Although atypical CNVs in the 16p11.2 interval are rare they are valuable in narrowing down the critical genomic interval. We detected one partial 110 kb duplication excluding *PRRT2* but encompassing *SPN*, *QPRT* and *C16orf54* in a patient who suffered from RE. The *QPRT* gene is of interest as it encodes an enzyme (quinolinate phosphoribosyltransferase), which converts quinolinic acid to NAD⁺ and thus detoxifies this excitotoxic compound (71,72). Intracerebral injection of quinolinic acid causes seizures in mice (73) and elevation of quinolinate levels in the human brain is associated with neurodegenerative disorders (74,75). While dosage differences in *QPRT* might explain the observed phenotype, this single, isolated rearrangement does not allow us to draw any firm conclusions, especially as we do not have any information on the CTS status of the single previously reported additional individual, who carried a partially overlapping 136 kb duplication, encompassing the *QPRT* gene (45).

Of interest, the detected borderline association of the 15q11.2 duplication with RE/ARE is supported when the comparison is made with its previously reported frequency in the general population (36) (6/281 cases versus 44/9841 controls, Fisher’s exact test, $P = 0.0025$). Hopefully, this finding will encourage further studies on larger RE/ARE cohorts to assess whether the 15q11.2 duplication really confers risk of RE/ARE.

In conclusion, we have identified 16p11.2 duplications as an important genetic risk factor for RE/ARE with 1.53% of patients carrying the variation. Our results suggest that the 16p11.2 duplication selectively confers susceptibility for RE/ARE but not for other common epilepsy syndromes, such as GGEs or TLE. An interesting but unresolved point concerns the role of additional genetic factors modulating the penetrance and the variable clinical features of the disease. Future studies on other well phenotyped cohorts could shed more light on these open issues.

MATERIALS AND METHODS

Study participants

In a multi-center effort 393 RE/ARE patients were recruited from Austria ($n = 160$), Germany ($n = 134$), Australia ($n = 78$) and Canada ($n = 21$). Diagnosis of RE was performed according to the International Classification of Seizures and Epilepsies (76). The various related types of atypical RE were diagnosed as specified previously (4,5,77). In the discovery cohort 98 of the patients were ascertained via multiplex-families with the minimum criteria of at least two affected siblings. The index case had to be diagnosed with RE or ARE, and the affected sibling(s) either with RE, ARE or the EEG trait only. All other 183 were RE/ARE patients recruited consecutively at the participating clinics, as soon as the diagnosis of RE/ARE was present, irrespective of their family history for seizures. The discovery set thus included a total of 281 unrelated patients of Caucasian ancestry affected by RE ($n = 230$) and ARE ($n = 51$) (165 males and 116 females), and 1512 unscreened German population controls (755 males and 757 females) examined by the same array type. The confirmation cohort consisted of 112 unrelated patients with RE ($n = 109$) and ARE ($n = 3$) (63 males and 49 females), from Australia ($n = 78$) and Austria ($n = 34$) ascertained through the diagnosis of RE/ARE irrespective of whether they had a family history of seizures. Additional SNP-array

data (Affymetrix Genome-Wide Human *SNP Array 6.0*) were obtained from 1408 GGE patients, 330 mTLE patients and 2256 German population controls (KORA: $n = 1250$, POGEN: $n = 1006$). We also studied 319 children carrying a 600 kb 16p11.2 rearrangement. These patients were assembled by screening neurodevelopmental disorders cohorts through a network of cytogenetic centers (44,45). For summarized description of all cohorts see Supplementary Material, Table S3. Written informed consent was obtained from all participating patients and, when appropriate, from both parents and adolescents. This study was approved by all respective local institutional review boards.

Genotyping, copy number variation detection and validation

CNVs were detected by high-density SNP genotyping arrays (Illumina HumanOmniExpress BeadChip) and subsequent CNV calling was carried out with the PennCNV software (78). CNVs were considered to match published recurrent CNVs if they overlapped at least 80% of the respective CNV-length. All called recurrent candidate CNVs were >350 kb in size and covered with 100–756 consecutive SNP-probes. Smaller CNVs of at least 50 kb nested within the recurrent candidate regions were considered if they included one of the previously proposed candidate genes (33,46). Genome-wide CNV screening beyond the six candidate regions was restricted to CNVs with a segment size >500 kb (79) and a minimum of 50 markers to achieve a high accuracy and reproducibility of CNV callings across different array platforms, laboratory sites and calling algorithms (80). CNVs were manually inspected in the Illumina Genome Viewer Software for the regional SNP heterozygosity state (BAF) and log₂ ratios of the signal intensities (LRR). One thousand five hundred and twelve controls genotyped on Illumina HumanOmniExpress BeadChip and 330 mTLE patients genotyped on IlluminaHap550 array were analyzed with PennCNV. Regions of CNVs detected in patients were manually inspected in the control samples for the presence of duplications and deletions. CNV calling of the 16p11.2 rearrangements in the 2256 German control subjects was performed by the Affymetrix SNP 6.0 array as previously reported (33,52). Recurrent CNV validation and segregation in families was performed with real-time quantitative PCR (qPCR) using TaqMan CNV probes (Supplementary Material, Table S5; Life Technologies, Darmstadt, Germany) and custom array-CGH for the 16p11.2 duplication (Agilent Technologies, Santa Clara, CA). This custom array contains about 544 probes in the 16p11.2 region (chr16:29500000–30200000, hg19) with an average probe spacing of 1.3 kb.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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